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Comparison of methyl eugenol metabolites, mitochondrial COI and rDNA sequences of *Bactrocera philippinensis* (Diptera: Tephritidae) with three other major pest species within the *dorsalis* complex

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Abstract

Males of the oriental fruit fly, *Bactrocera dorsalis* (Hendel), and some of its sibling species show a strong affinity to methyl eugenol (ME). Methyl eugenol ingested by male flies is biotransformed in the crop to two ME-metabolites that eventually accumulate in the rectal gland, which is known to serve as a reservoir for *B. dorsalis* sex pheromones. Upon ME-feeding, males of laboratory and wild *B. philippinensis* Drew and Hancock selectively accumulated two metabolites, 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol, in the rectal gland, as was seen in *B. dorsalis sensu stricto*, *B. invadens* Drew, Tsuruta and White and *B. papayae* Drew and Hancock. Phylogenetic analysis of COI and rDNA sequence data of these four taxa also revealed a tight relationship among *B. philippinensis, B. dorsalis s.s., B. invadens* and *B. papayae* (all four are members of the *dorsalis*-species complex). This result corroborates the pheromone analysis. The usefulness of pheromonal analysis as a chemotaxonomy tool to complement molecular and other analyses in the differentiation of closely related sibling species within the *Bactrocera dorsalis* complex, where using morphological characters had been inadequate, is highlighted.

Key words: *Bactrocera philippinensis* – *Bactrocera dorsalis* species complex – pheromone – methyl eugenol – mitochondrial DNA

Introduction

Currently, there are 75 species within the *Bactrocera dorsalis* Hendel species complex (Diptera: Tephritidae) (Clarke et al. 2005), an increase of over twenty species from the 52 species revised by Drew and Hancock (1994). Of these, 26 species are known to be responsive to the male attractant methyl eugenol (ME), including *B dorsalis s.s.*, *B. invadens* Drew, Tsuruta and White, *B. papayae* Drew and Hancock (synonym *B. dorsalis sensu lato* – see discussion), and *B. philippinensis* Drew and Hancock; all of which are considered serious and highly invasive fruit pests (Clarke et al. 2005; Tan et al. 2011).

Bactrocera philippinensis is recorded as an endemic and notable pest species in the Philippines and has a distinct geographical range from *B. dorsalis s.s.* (Clarke et al, 2005). In Palau, it was first recorded and mistakenly identified as *B. dorsalis* in September, 1996 but confirmed as *B. philippinensis* five years later (http://www.spc.int/Pacifly/Species_profiles/B_philippinensis.htm).

Females of *B. dorsalis* from different localities/countries have significantly different average aculeus length in descending order India>Thailand>Hawaii>Taiwan (Mahmood 1999). Many females from these places, except Taiwan, have aculeus length that overlaps with that of *B. papayae* and *B. philippinensis* (Mahmood 2004). This together with other morphometric data led Mahmood (2004) to conclude that “no tenable method has been found to separate specimens of *B. dorsalis* from *B. papayae*

and *B. philippinensis* using external morphological characters".

It was reported that females of *B. philippinensis* may be differentiated from *B. papayae* by having shorter length of scales found on the distal end of the eversible membrane of the ovipositor (Drew and Hancock, 1994). Nevertheless, recent work via electron scanning microscopy by Mahmood (2004) showed no difference in the length of scales on the distal end of the eversible membrane of the ovipositor between *B. papayae* and *B. philippinensis*. These inconsistencies show that the morphological characters and their morphometrics may be more consistent with population-level, rather than species-level, variations. Furthermore, phylogenetic studies have shown that *B. philippinensis* is monophyletic with respect to *B. dorsalis s.s.* and *B. papayae* (Armstrong and Cameron 2000; Muraji and Nakahara 2001; Zhang et al. 2010; Krosch 2012a, b). Therefore, it is of great importance and urgency to understand the phylogenetic relationship using mitochondrial genes sequences in conjunction with other characters such as sex pheromone profiles, which would provide a distinctive feature on diversification among the sibling species (Tan et al. 2011).

Like other notorious *Bactrocera* pest species, males of *B. philippinensis* are strongly attracted to and compulsively feed on ME, which is found naturally in over 450 plant species from 80 families spanning across 38 orders (Tan and Nishida 2012). Consumption of ME significantly improves male mating performance and competitiveness of *B. dorsalis s.s.* (Shelly and Dewire 1994, Shelly 2000; Tan and Nishida 1996; Shelly and Nishida 2004; Orankanok et al. 2011), *B. papayae* (Tan and Nishida 1996, 1998), *B. carambolae* (Wee et al. 2007). ME acts as a sex pheromone

precursor in *B. dorsalis s.s.* and *B. papayae* in which ME is biotransformed to (*E*)-coniferyl alcohol (E-CF) and 2-allyl-4,5-dimethoxyphenol (DMP) (Fig. 1) (Nishida et al. 1988; Tan and Nishida 1996, 1998). The two volatile phenylpropanoids, temporarily stored in the rectal gland and emitted at dusk prior to courtship, were shown to act as sex pheromone that attracted conspecific females of *B. dorsalis s.s.* (Nishida et al. 2000) and *B. papayae* (Tan and Nishida 1996, 1998; Hee and Tan 1998; Khoo et al. 2000). The same ME metabolites, E-CF and DMP, were also detected in the male rectal gland of *B. invadens*, a highly invasive species in the African continent (Tan et al. 2011).

After consuming ME, irradiated males of *B. philippinensis* showed a mating advantage compared with males never exposed to the attractant (Shelly et al. 1996). Since the rectal/sex pheromone volatiles have not been identified in this species, we aim to determine the fate of ME after consumption by males and to examine the phylogenetic relationship of *B. philippinensis* in relation to three other putative species, *B. dorsalis s.s., B. invadens* and *B. papayae,* within the *B. dorsalis* complex, by using the mitochondrial COI and rDNA sequences. Lately, a study showed that *B. dorsalis s.s., B. papayae* and *B. philippinensis* represent one biological species based on independent data sets on mitochondrial DNA and wing shape (Schutze et al. 2012).

Materials and methods

Insects

Bactrocera philippinensis, originally from Guimaras, Philippines, and positively identified by R.A.I. Drew in 2009, was cultured in the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria. Due to strict quarantine restrictions, experiments were conducted exclusively on sexually mature males (21-25 day-old) at the Seibersdorf laboratory.

Chemical analysis

Gas chromatography-mass spectrometry (GC-MS) was performed with an Agilent 5975 inert XL MSD mass spectrometer (electron impact ionization at 70 eV) linked to an Agilent 6890 gas chromatograph equipped with a HP-5MS column (28 m × 0.25 mm, 0.25 μm film thickness), using helium as a carrier gas and programmed from 60°C (1 min holding) to 280°C at a rate of 10°C/min. The GC quantification was carried out with an HP 5890 series II plus using a HP-1 column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temperature was programmed from 60^{o}C (2 min holding) to 240^{o} C at a rate of 10 ^{o} C /min using 1-hexadecanol (Wako Pure Chemical Industries, Japan) as an internal standard. The carrier gas was helium; and detection was by a flame ionization detector (FID).

Feeding test and rectal sample preparation

Males of *B. philippinensis* (21-25 day old; approximately the 50th generation) were allowed to feed on methyl eugenol (Aldrich Chemicals Co., USA) impregnated into small filter paper discs (Advantec, antibiotic test disc, thick, 8 mm diameter) (10

µl/disc) *ad libitum* for *ca.* 15 min and were then kept in a cylindrical plastic rearing cage (15 cm diameter x 20 cm height) with a sufficient amount of adult food (sugar-yeast hydrolysate mixture) and water at 24-30°C under ambient light conditions. Rectal glands were dissected from the males at 6, 12, 24 and 48 hours following ME feeding. Each extracted gland was individually preserved in 250 µl of redistilled ethanol in a 1 ml screw-capped glass vial, and then, kept in a freezer at -20^{o}C until chemical analysis. The significant differences of the contents between DMP and E-CF were analyzed by Wilcoxon signed-ranks test.

Field trapping of wild male flies

Wild males were captured using ME-baited sticky traps at Castillejos, Zambales, Philippines (Luzon Island) in 2007. The whole fly was carefully extracted from the sticky trap and preserved individually in a vial containing redistilled ethanol (ca 250µl). Each of the specimens was subjected to morphological identification as *B. philippinensis* before their rectal glands were extracted individually. A total of 18 males were examined chemically.

Identification and quantification of ME-metabolites in male rectal gland

Chemical identification was based on comparison of their retention time and mass fragment pattern with those of authentic chemicals. Quantification of the ME-metabolites was conducted as previously described by Tan et al. (2011).

Molecular cloning and sequence analysis

DNA extraction from individual adults, PCR amplification, and sequence analysis were performed as described by Tan et al. (2011). PCR amplifications were performed for the mitochondrial cytochrome oxidase subunit I (COI) gene and rDNA containing a part of the 16S rRNA gene, the $tRNA^{val}$ gene, and a part of the 12S rRNA gene. The forward (f) and reverse (r) primer pairs were as follows: for COI, f (5'-ATTTATAATGTAATTGTAACAGC-3') and r (5'-GAAGTATTTAARTTTCGRTCTG-3'); and for rDNA, f (5'-TTCAGTGGGCAGGTCAGACT-3') and r (5'-ATATGCACACATCGCCCGTC-3'). The PCR products were cloned into the pGEM-T Easy vector (Promega, WI, USA) and sequences of the clones were determined using T7 and SP6 universal primers. The DNA sequence data have been deposited in the DDBJ/EMBL-Bank/GenBank as listed in Table 1.

Phylogenetic analysis

The DNA sequences were determined for *B. dorsalis s.s.*, *B. invadens*, *B. papayae*, *B. philippinensis* (these species are taxonomically real, yet biologically dubious, entities) and another sibling species of the *dorsalis* complex, *B. carambolae*, which has a distinctive sex pheromone profile compared with the four species investigated; and two unrelated ME-responsive species, *B. correcta* and *B. zonata* were included in the phylogenetic analysis to provide an insight into the relationship between sex pheromone diversification and genetic distance amongst the fruit fly species. The corresponding

genes of the complete mitochondrial genome of *B. cucurbitae*, a non-ME responsive *Bactrocera* species but a cue-lure- and raspberry ketone-responsive pest (JN635562), were used as out groups.

Sequence alignment was performed using the program Clustal W 1.8.3. The phylogenetic trees were generated by aligning the determined sequences excluding the primer regions together with those of Tokushima et al. (2010) and Tan et al. (2011) using the corresponding genes of *B. cucurbitae* as an out group. The maximum likelihood (ML) method was conducted by MEGA 5 (Tamura et al. 2011). Parameters were based on the General Time Reversible model with among-site rate heterogeneity according to a Gamma distributed with Invariant sites (G+I).

Results

Identification and accumulation of methyl eugenol metabolites in the rectal glands

Two ME-derived phenylpropanoids, DMP and E-CF (Fig. 1), were detected from rectal glands of male *B. philippinensis* following ME feeding. The identity of the metabolites were confirmed by GC-MS data as follows.

DMP (2-allyl-4,5-dimethoxyphenol). GC: RI (relative retention index on HP-5MS): 1630. MS: *m/z* (%) 194 (100, M^+), 179 (87), 163 (10), 151 (16), 136 (7), 133 (8), 123 (32), 119 (9), 105 (7), 95 (10), 91 (23), 79 (11), 77 (16), 69 (27), 53 (11).

E-CF ((*E*)-Coniferyl alcohol). GC: RI 1748. MS *m/z* (%): 180 (71, M^+), 162 (10), 147 (13), 137 (100), 131 (15), 124 (52), 119 (25), 109 (13), 103 (18), 91 (39), 77 (19), 65

(14), 55 (12).

Figure 2 shows the quantities of DMP and E-CF present in the rectal gland of male *B. philippinensis* at 6, 12, 24 and 48 h after feeding on ME. Both phenylpropanoids increased in quantity with time and peaked at 24 h, and thereafter a decrease was observed at 48 h post feeding. The total quantity of the two compounds was approximately 20 µg/gland at 24 h post ME feeding. The individual contents of DMP and E-CF in the rectal gland of the males were variable at any given time after feeding on ME. While no significant difference in contents between DMP and E-CF was detected at 6h and 48h post ME feeding, the contents of E-CF were significantly higher than those of DMP at 12h and 24h post ME feeding (Wilcoxon signed-ranks test, 12h: $Z = -2.24$, $N = 8$, $P = 0.025$; 24h: $Z = -1.99$, $N = 6$, $P = 0.046$).

Phenylpropanoid contents in the wild males

Some of the wild *B. philippinensis* male flies captured by ME-baited sticky traps in Luzon, Philippines, lacked both DMP and E-CF or either one of the metabolites. Hence, it contributed to the high variation in total contents per fly [Mean content ± S.E.: DMP 4.7 ± 6.7 µg/gland; E-CF, 1.6 ± 3.5 µg/gland]. The ratio of DMP and E-CF detected in the rectal glands varied significantly for wild males with a bias towards DMP, while laboratory-raised males have less variation (Fig. 3).

Phylogenetic analyses of *B. philippinensis* in relation to other *Bactrocera* species

The fragments of COI and rDNA were amplified by PCR, and the length of

the resulting nucleotide was 497 bp and from 884 to 888 bp, respectively. Both COI and rDNA analyses show that a lineage comprising of *B. philippinensis*, *B. dorsalis s.s.*, *B. invadens*, *B. papayae* and *B. carambolae* was quite distinct from the two individual lineages derived from *B. zonata* and *B. correcta* (Fig. 4). These phylogenetic trees based on the mitochondrial genes clearly show that *B. philippinensis* belongs to the same clade as the *B. dorsalis s.s.* as well as *B. invadens*, *B. papayae* and *B. carambolae*.

Discussion

After consuming ME, two rectal (sex pheromone) volatile components, DMP and E-CF, were detected in *B. philippinensis* males. These phenylpropanoid components were also previously detected in the other three closely related species of the *B. dorsalis* complex after pharmacophagy of ME, namely *B. dorsalis s.s.* (Nishida et al., 2000) and *B. papayae* (Tan and Nishida 1996), and *B. invadens* (Tan et al. 2011). But it was found to differ partially from its sibling species, *B. carambolae* (Wee and Tan 2007), and totally from other ME-responsive species that are not within the *dorsalis* complex – namely *B. correcta* and *B. zonata* (Tokushima et al. 2010, Tan et al. 2011). Insect sex pheromone is often highly species-specific and often serves as an important chemical cue, although not solely responsible, for mate recognition and helps to delimit gene flow among different populations through different blend, ratios and isomers, as in the European ermine moth, *Yponomeuta* spp. (Löfstedt et al. 1991). Therefore, the presence of identical sex pheromone profiles of DMP and E-CF among the four species strongly

indicates that the species belong to the same biological species because they possess the same 'chemical' language for sexual communication. It is speculated that if their movements had not been restricted by international trade and quarantine restrictions, they would have been able to 'meet' and mate amongst themselves without difficulty. Previous mating study conducted in the Philippines also showed that there is high reproductive compatibility between *B. philippinensis* and *B. dorsalis s.s.* (Medina et al. 1988). Additionally, mating compatibility studies conducted by A. Jessup, in the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, have shown that *B. invadens* and *B. philippinensis* were able to interbreed readily with *B. dorsalis s.s.* and reproduced ''highly fertile F_1 and F_2 hybrids'' (A. Jessup, IAEA — personal communication 2010, 2011).

Similarly, the ability to interbreed between *B. dorsalis s.s.* and *B. papayae* and yield viable offspring up to F_3 (Tan 2003) indicated that the two sibling species are not distinct biological species. This is further supported by the genetic evidence that one of three actin gene alleles in *B. dorsalis s.s.* and *B. papayae* – allele BdorA1 and allele BpapA2, respectively, have identical DNA sequence (Naeole and Haymer 2003). Hence, *B. papayae* has been referred to as *B. dorsalis sensu lato*. Recent population genetic analysis also corroborated these species as one and the same biological species (Schutze et al. 2012; Krosch et al. 2012a).

Similar trend of variations in the ratio of sex pheromone components was also reported in *B. papayae* males whereby laboratory raised *B. papayae* males produced higher amount of E-CF than DMP and the vice versa was seen in the wild conspecific

males (Wee and Tan 2001). Prolonged inbreeding in enclosures may induce insects to adapt to laboratory conditions that may affect changes in the pheromone titers (Giebultowicz et al. 1992; Raina et al. 1989). It was also suggested that the production and ratio of DMP to E-CF may be indirectly related to the contemporary needs of individual flies, as DMP was demonstrated to be a much weaker sex attractant than E-CF, for *B. papayae* females (Hee and Tan, 1998). In addition, DMP was observed to deter birds better than ME, followed by E-CF (Nishida and Fukami, 1990). Thus, it was hypothesized that the E-CF as a sex pheromone component is more crucial than allomone component (DMP) in the laboratory where the need to deter predators is greatly reduced hence the consistence in higher E-CF to DMP ratio. Additionally, the variations may indicate that the ratio of the sex pheromone components may not be a good indicator for distinguishing tephritid species as the ratio may be dependent on natural sources (often unknown) of the chemicals which the males feed on, as found in certain *Bulbophyllum* orchid floral fragrance or secretion that contains either one or both of the sex pheromone components besides ME (Tan et al. 2002, 2006).

The wild *B. philippinensis* males that did not possess ME-metabolites may indicate that they had either no opportunity to feed on natural sources of ME or likely just attained sexual maturity to be attracted for the first time to ME to initiate pharmacophagy. This phenomenon was also shown in wild *B. papayae* (Nishida et al. 1988; Tan and Nishida 1996, 1998; Wee and Tan 2001). Further work is needed to clarify the nature of pheromone acquisition, sequestration, emission and behavioral effects on the courtship sequences in *B. philippinensis*, particularly under its natural

environment, in comparison with those studies conducted for other sibling species.

Phylogenetic analyses on *B. philippinensis* from Guimaras and Luzon, Philippines — based on the comparison of nucleotide sequences in the mitochondrial genes, COI and rDNA — showed that the species belongs to the same species clade as *B. carambolae, B. dorsalis s.s., B. invadens* and *B. papayae* but differs from the *B. zonata* species complex which is consistent with previous rDNA analysis by Muraji and Nakahara (2001) and Krosch et al. (2012a). Similar phylogenetic relationship based on the two mitochondrial genes was obtained for seven pest sibling species, namely *B. carambolae, B. dorsalis s.s., B. kandiensis, B. musae* (Tryon), *B. occipitalis, B. papayae* and *B. philippinensis*, which appeared to have a common ancestor, within the *B. dorsalis* complex (Zhang et al. 2010). This further shows that *B. philippinensis* is indeed very closely related to the other ME-responsive sibling species within the *B. dorsalis* species complex. Furthermore, a recent phylogenetic analysis using fruit flies collected in the Ryukyu Islands, Taiwan, and the Philippines, and from fruits imported from the Philippines and China intercepted at Narita International Airport showed fruit flies possessing one of five banding patterns were grouped as *B. philippinensis* based on the sequence of 1,138 bp mitochondrial DNA fragment from 16S to 12S rDNA (Muraji et al. 2008). This led to an inference that the fruit flies collected in Sakishima region, Japan, might have flown in directly from the Philippines.

In conclusion, the above evidence in this paper – chemical ecology and chemotaxonomy as well as DNA analyses together with other evidence from different perspectives, i.e., mating compatibility and hybrid viability, as well as molecular

analyses, strongly indicate that *B. philippinensis* and its three very close sibling species, namely – *B. dorsalis s.s.*, *B. invadens* and *B. papayae*, belong to the same biological species. Therefore, based on the integrative and comprehensive approach, these four species, currently discriminated by indistinct and variable morphological characters that are unreliable for differentiating the sibling species (Mahmood 2004), should be recognized taxonomically as either different populations or strains of a single biological species, *B. dorsalis*. More importantly, they should not remain as the status quo of four distinct putative species – currently based solely on indistinct morphological characters, which have caused much confusion worldwide in the identification of the sibling species in quarantine surveys and interceptions, as well as untold economic losses due to quarantine restrictions in international trade, especially for third world or developing countries.

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Legends

Fig. 1 Chemical structures of methyl eugenol (ME) and its metabolites, 2-allyl-4,5-dimethoxyphenol (DMP) and *E*-coniferyl alcohol (E-CF) sequestered by *Bactrocera philippinensis* males post feeding on ME.

Fig. 2 Contents (mean ± S.E.) of 2-allyl-4,5-dimethoxyphenol (DMP: white) and *E*-coniferyl alcohol (E-CF: gray) sequestered by *Bactrocera philippinensis* males 6, 12, 24 and 48 h post feeding on methyl eugenol (N = 8 for 6, 12 and 48 h; N = 6 for 24 h).

Fig. 3 Contents of 2-allyl-4,5-dimethoxyphenol (DMP) and *E*-coniferyl alcohol (E-CF) accumulated in the individual rectal glands of wild *Bactrocera philippinensis* males captured in a field site in Luzon Island, Philippines (black) (N = 18) and laboratory males artificially fed on methyl eugenol (48 hr post treatment) (circle) (N = 8).

Fig. 4 Phylogenetic trees constructed by maximum likelihood (ML) analysis. The values shown at the nodes of the branches are bootstrap values (%) from 1000 replicate bootstrap samplings. Branch length is proportional to ML estimated genetic distances. The scale bars indicate the number of nucleotide substitutions per site. GenBank accession numbers are listed in Table 1. (A) Phylogenetic tree

deduced from COI sequences. (B) Phylogenetic tree deduced from rDNA sequences.

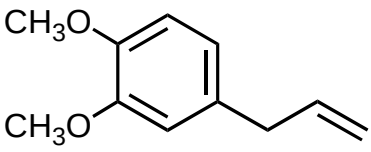
Table 1. List of the Genbank accession numbers of *Bactrocera* species analysed

Accession number	Gene	Spiecies	Geographic origin (year)	References
AB720885	COI	*B. philippinensis*	IAEA (Vienna) –A, origin: Guimaras, PH (2010)	This study
AB720891	rDNA	*B. philippinensis*	IAEA (Vienna) –A, origin: Guimaras, PH (2010)	This study
AB720886	COI	*B. philippinensis*	IAEA (Vienna) –B, origin: Guimaras, PH (2010)	This study
AB720892	rDNA	*B. philippinensis*	IAEA (Vienna) –B, origin: Guimaras, PH (2010)	This study
AB720882	COI	*B. philippinensis*	Wild-A, Luzon, PH (2007)	This study
AB720893	rDNA	*B. philippinensis*	Wild-A, Luzon, PH (2007)	This study
AB720883	COI	*B. philippinensis*	Wild-B, Luzon, PH (2007)	This study
AB720894	rDNA	*B. philippinensis*	Wild-B, Luzon, PH (2007)	This study
AB720884	COI	*B. philippinensis*	Wild-C, Luzon, PH (2007)	This study
AB720895	rDNA	*B. philippinensis*	Wild-C, Luzon, PH (2007)	This study
AB568103	COI	*B. dorsalis s.s.*	Laboratory, Bangkok, TH (2009)	Tan et al. (2011)
AB568107	rDNA	*B. dorsalis s.s.*	Laboratory, Bangkok, TH (2009)	Tan et al. (2011)
AB720887	COI	*B. dorsalis s.s.*	Wild, Oahu, US (2002)	This study
AB720896	rDNA	*B. dorsalis s.s.*	Wild, Oahu, US (2002)	This study
AB568104	COI	*B. invadens*	IAEA (Vienna), origin: KE (2009)	Tan et al. (2011)
AB568108	rDNA	*B. invadens*	IAEA (Vienna), origin: KE (2009)	Tan et al. (2011)
AB720888	COI	*B. papayae*	Wild, Penang, MY (2007)	This study
AB720897	rDNA	*B. papayae*	Wild, Penang, MY (2007)	This study
AB720889	COI	*B. carambolae*	Wild, Selangor, MY (2008)	This study
AB720898	rDNA	*B. carambolae*	Wild, Selangor, MY (2008)	This study

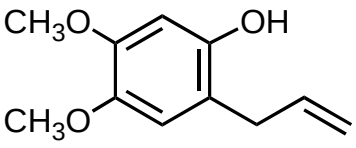
AB720890	COI	*B. tryoni*	IAEA (Vienna), origin: Queensland, AU (2007)	This study
AB720899	rDNA	*B. tryoni*	IAEA (Vienna), origin: Queensland, AU (2007)	This study
AB720881	COI	*B. correcta*	Wild, Bangkok, TH (2007)	This study
AB569585	rDNA	*B. correcta*	Wild, Bangkok, TH (2007)	Tokushima et al. (2010)
AB721013	COI	*B. correcta*	Wild, Guava orchard[a], TH (2009)	This study
AB569586	rDNA	*B. correcta*	Wild, Guava orchard [a], TH (2009)	Tokushima et al. (2010)
AB568102	COI	*B. correcta*	Laboratory, Bangkok, TH (2009)	Tan et al. (2011)
AB568106	rDNA	*B. correcta*	Laboratory, Bangkok, TH (2009)	Tan et al. (2011)
AB568105	COI	*B. zonata*	Laboratory, MU (2009)	Tan et al. (2011)
AB568109	rDNA	*B. zonata*	Laboratory, MU (2009)	Tan et al. (2011)

[a] 14°07'55.559" North; 100°48'28.762" East

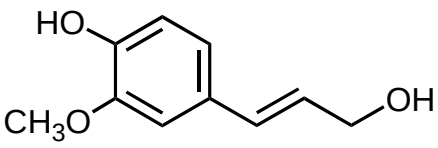
Fig. 1



Methyl eugenol (ME)



DMP



E-CF

Fig. 2

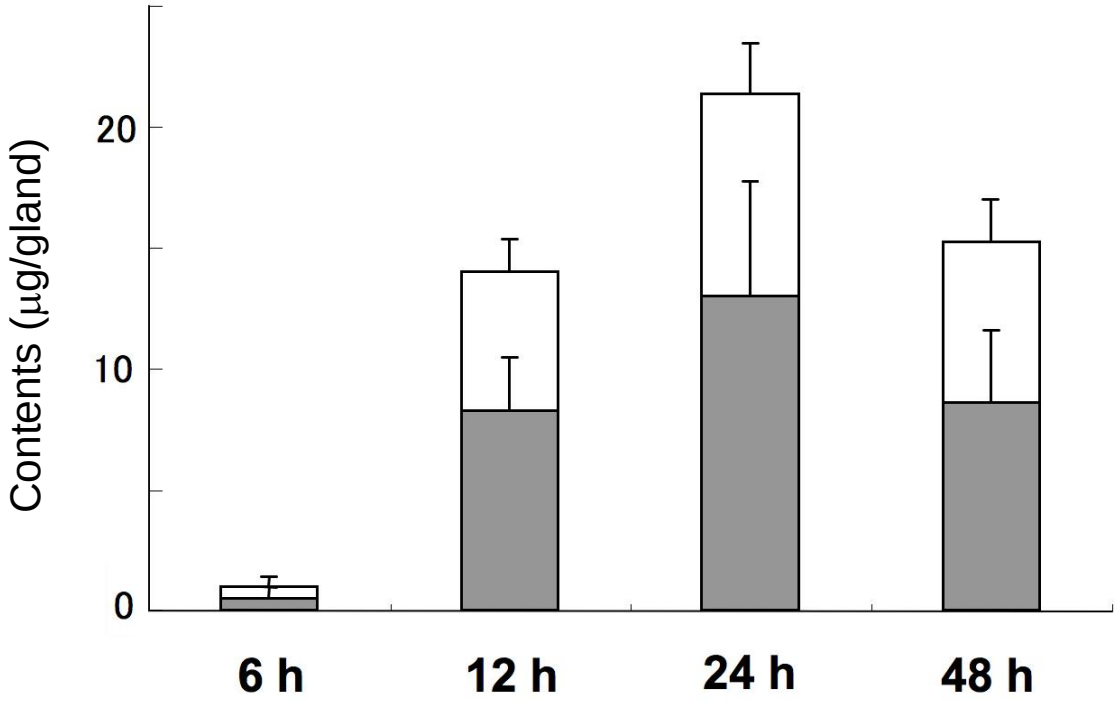


Fig. 3

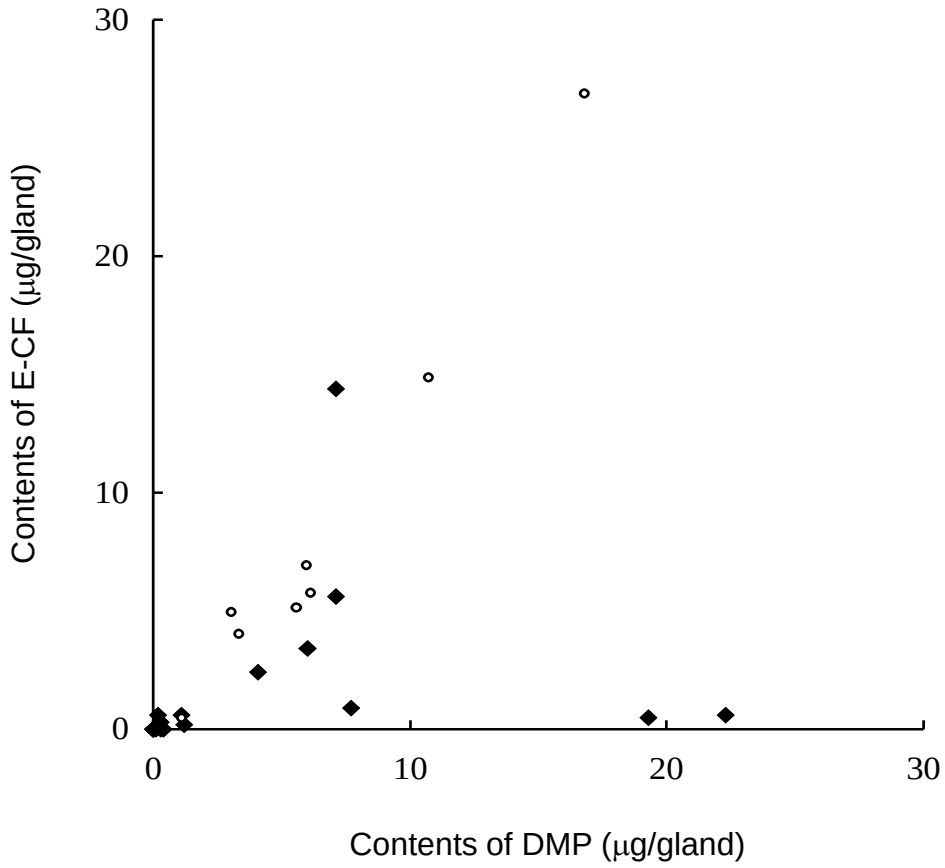


Fig. 4